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PRINCIPAL INVESTIGATOR(S): Michael F. Clarke, M.D.

CONTRACTING ORGANIZATION: University of Michigan
Ann Arbor, Michigan 48109

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Breast cancer is the second leading cause of cancer death among American women, with over 170,000 new cases and 50,000 deaths each year. Despite advances in detection and treatment, mortality from these diseases remains high. Traditional modes of treatment including radiation therapy, chemotherapy, and hormonal therapy have been useful but are limited by the emergence of treatment-resistant cancer cells. Clearly, new approaches are needed to treat these diseases. This project is designed to develop novel approaches to detect breast cancer cells that contaminate peripheral blood and bone marrow, and to remove such contaminating cells. An RT-PCR assay has been developed to detect breast cancer cells, and a novel gene therapy vector has been developed to kill contaminating cancer cells. Blood and bone marrow samples obtained from patients with breast cancer are being collected. These samples will be analyzed to determine whether the K19 RT-PCR assay can be used to predict outcome. Next, a gene therapy vector, the bcl-xs adenovirus, has been developed. This vector has promise as a therapeutic agent for the treatment of breast cancer. With additional support from the National Cancer Institute, this virus is undergoing toxicology testing in order to obtain FDA approval for human clinical trials for the treatment of breast cancer.

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INTRODUCTION

Breast cancer is the second leading cause of cancer death among American women, with over 170,000 new cases and 50,000 deaths each year. Despite advances in detection and treatment, mortality from these diseases remains high. Traditional modes of treatment including radiation therapy, chemotherapy, and hormonal therapy have been useful but are limited by the emergence of treatment-resistant cancer cells. Clearly, new approaches are needed to treat these diseases.

One of the more promising approaches for the treatment of metastatic breast cancer is high dose chemotherapy. Breast cancer is susceptible to chemotherapy in a dose dependent manner. The major dose limiting toxicity of many effective chemotherapeutic agents is hematopoietic toxicity. To overcome this obstacle, autologous bone marrow or peripheral blood stem cells are harvested from the patient prior to the administration of the high dose chemotherapy, and then reinfused after the chemotherapy has been excreted and/or metabolized. Although the initial clinical trials of such strategies are promising, there are other obstacles that need to be overcome to optimize results. The agents used for the systemic treatment of this disease need to be improved. Additionally, bone marrow transplantation for solid tumors such as breast cancer is complicated by the fact that these cancers frequently metastasize to the bone marrow.

To date, two significant advances have resulted from this proposal. A sensitive assay has been developed to identify breast cancer cells in the bone marrow and the peripheral blood. Next, an adenovirus vector has been developed that is selectively lethal to breast cancer cells, but not normal cells. The results of preclinical studies with this virus have been successful. The National Cancer Institute (NCI) has decided to hold an IND for this virus, and toxicology testing is underway in order to obtain FDA approval for clinical trials. This virus should enter clinical trials for the treatment of breast cancer sometime in the next two years.

BODY

In the first two years of this grant, significant progress in completion of the goals of this grant has been made.

Task 1. To test the hypothesis that women with poor prognostic indicators are more likely to present with the presence of blood and/or bone marrow micrometastases is ongoing with sample collections.

-1A. Sample collection and PCR assays. To date, we have collected blood samples from 123 patients, including 20 patients with stage I or II cancer, nine with stage III , and 123 with stage IV breast cancer. We plan to increase the number of samples obtained from patients with the stage I, II and III breast cancer.

-1B. Clinical follow-up. Follow-up of the outcome of the stage I, II and III prognosis patients will begin when more samples are obtained from this group of patients. See enclosed informed consent and IRB approval for continued sample collection.

Task 2. To use a PCR based assay to detect the mammary cell specific keratin-19 mRNA and evaluate the presence of occult breast cancer cells in patients undergoing BMT is progressing well, indeed ahead of schedule, and two manuscripts have been published that address this task.

-1A. To determine the relative frequency of tumor contamination of marrow versus peripheral blood stem cell harvests. To date, we have collected samples from 59 patients that have undergone BMT. Forty seven samples are from peripheral blood stem cell harvests, and 13 are from bone marrow harvests (one patient had both aphoresis and bone marrow). To date, 20% of the peripheral blood samples have been positive, and 46% of the bone marrow samples have been positive. At the time this grant was written, bone marrow harvest was the source of the hematopoietic cells used for rescue from high dose chemotherapy. Now, peripheral blood stem cell harvests are used exclusively for rescue from high dose chemotherapy. Since patients routinely get a diagnostic bone marrow prior to high dose chemotherapy, we have amended our protocol in order to analyze peripheral blood, the diagnostic bone marrow specimen, and the aphoresis product for K19.

-1B. Correlation of PCR results with clinical outcome. There are not yet enough samples obtained from patients with stage I-III breast cancer. This is also the case with breast cancer patients undergoing high dose chemotherapy. The positive rate in peripheral blood stem cell harvests is lower than that seen in bone marrow. Since peripheral blood stem cell harvests is used, only about 20% of the patients are K19 positive. Only about 20% of such samples are PCR positive for cancer. Therefore, the sample size is still too small to make any clinical correlation's at this time.

-1C. Evaluate the efficacy of BM and stem cell culture purging techniques to eliminate breast cancer cells.

We have published a manuscript that describes a novel method for purging contaminating cancer cells from bone marrow hematopoietic stem cells. Many cancers overexpress a member of the *bcl-2* family of inhibitors of apoptosis. To determine the role of these proteins in maintaining cancer cell viability, an adenovirus vector that expresses *bcl-x$_S$*, a functional inhibitor of these proteins, was constructed. Even in the absence of an exogenous apoptotic signal such as x-irradiation, this virus specifically and efficiently kills carcinoma cells arising from multiple organs including breast, colon, stomach, and neuroblasts. In contrast, normal hematopoietic progenitor cells and primitive cells capable of repopulating immune-deficient SCID mice were refractory to killing by the *bcl-x$_S$* adenovirus. This vector may prove useful in killing cancer cells contaminating the bone marrow of patients undergoing autologous bone marrow transplantation. (appendix, manuscript #1).

Since the last report, significant progress in the purging of breast cancer cells has been made. We have further defined the use of adenovirus suicide vectors for killing breast cancer cells that contaminate the bone marrow of patients with breast cancer. Last year, we found that a pure population of mouse hematopoietic stem cells are not transduced by an adenovirus vector. We have extended this observation and now show that human hematopoietic stem cells are also not transduced by such vectors. The National Cancer Institute has now decided to do toxicology testing of the *bcl-xs* adenovirus to obtain FDA

approval for use of the virus in human clinical trials. We envision such trials to begin within the next two years.

BACKGROUND

The use of autologous bone marrow transplant (BMT) as a part of cancer treatment has increased dramatically in recent years. For example, autologous BMT often is used to protect patients from the hematopoietic toxicity of high dose chemotherapy in the treatment of breast tumors [1,2]. However, the success rate for this treatment regimen is jeopardized by contamination of the autologous marrow with low numbers of tumor cells [3-6]. Thus, the ability to selectively kill, or purge contaminating tumor cells in the marrow *in vitro* prior to autologous transplant, could significantly improve the chances of long term survival.

The most common technique of purging tumor cells from bone marrow relies upon monoclonal "anti-tumor" antibodies to immunologically remove tumor cells from a suspension of marrow cells [7-9]. However, the success of this approach depends on the availability of well characterized antibodies capable of detecting each tumor cell phenotype. This method of purging rarely removes all tumor cells, and requires repeated purging cycles which can decrease hematopoietic cell viability [10]. Moreover, the specific antigens are rarely "tumor cell specific", often being found on normal cells, albeit at low antigen density. Other purging methods, such as photo-sensitization agents or chemotherapy, also can cause significant hematopoietic toxicity [1,11,12].

Until recently, investigators thought that cancer treatments based on chemotherapy, or radiation therapy, exerted their tumor-killing specificities based on the different sensitivities of rapidly dividing (versus quiescent) cells to these agents. Recent evidence suggests that radiation and many chemotherapy agents specifically kill tumor cells, while sparing normal cells, by triggering of the Programmed Cell Death (PCD) pathway [13-15]. The induction of PCD is impeded by genes such as *bcl-2*, and its presence in cancer cells thus reduces the efficiency of conventional cancer therapy. Mechanistically, *bcl-2* does not appear to be mitogenic or transforming, but it cooperates with c-myc, and members of the ras family, to cause transformation [16-18]. Additionally, *bcl-2* acts to inhibit apoptosis induced by p53, *myc*, chemotherapy, and ionizing radiation [19-23]. Members of this gene family have been implicated in the progression of a large number of human solid tumors, including lymphomas, cancers of the breast, lung, and prostate, as well as neuroblastoma [24]. These observations suggest that suppression of *bcl-2* expression, using gene therapy methods, would be a valuable tool in cancer treatment by increasing the susceptibility of tumor cells to existing chemotherapeutic and radiation treatments.

Other members of the *bcl-2* gene family have recently been isolated and partially characterized. A *bcl-2* homologue, *bcl-x*, gives rise to two mRNA species through alternative splicing. One of these, *bcl-x_L*, functions in a manner similar to *bcl-2*, and inhibits apoptosis. The other, *bcl-x_S*, functions as a repressor to *bcl-2* and acts to promote apoptosis. We recently demonstrated that adenoviral-mediated overexpression of *bcl-x_S* inhibited the anti-apoptotic role of *bcl-2* and induced PCD in a variety of primary tumors and tumor cell lines [25]. This PCD was augmented by, but not dependent upon, the tumor suppresser p53. In contrast, normal bone marrow hematopoietic stem cells resisted *bcl-x_S*-adenovirus induced PCD apoptosis. We postulated that the resistance of hematopoietic stem cells to the *bcl-x_S* adenovirus was due to the lack of expression of adenovirus transgenes in

hematopoietic stem cells or alternatively to the inability of *bcl-x_S* to induce cell death in these cells. We report that murine hematopoietic stem cells resist expression of an adenovirally transduced gene. Moreover, a *bcl-x_S* adenovirus works synergistically with a Herpes virus TK adenovirus to specifically purge tumor cells from *in vitro* hematopoietic cultures, with the preservation of transplantable stem cells. Thus, these results indicate that adenovirus vectors fail to transduce genes into early hematopoietic stem cells. Therefore, this suggests that adenovirus vectors encoding suicide genes such as *bcl-x_S* or Herpes virus TK would preferentially kill the contaminating tumor cells derived from epithelial tissues found in bone marrow cell populations, thus serving as an excellent means of marrow purging.

MATERIALS AND METHODS

Primary Bone Marrow Cells. Human bone marrow cells were obtained from the posterior iliac crest of normal volunteers following informed consent using a protocol approved by the University of Michigan Human Institutional Review Board. Bone marrow mononuclear cells were separated by density gradient centrifugation on Ficoll-Paque (1.077 g/mL; Pharmacia). Cells were collected from the interface and washed three times in Iscove's modified Dulbecco's medium (IMDM; Gibco). Cells were then counted and divided for appropriate infection conditions. To isolate murine stem cells, bone marrow was harvested from the femur and tibia of c57black/Ka mice congenic for Thy 1.1. Bone marrow cells were stained with anti-Thy 1.1, anti-Sca-1, anti-Kit, and an anti-Lin cocktail consisting of anti-FcgII/FcgIIb, anti-Ly5.2, anti-CD3, anti-CD4, Anti-CD5, anti-CD8, anti-erythrocyte-specific antigen, anti-B220, anti-Gr-1, and anti-Mac-1[26]. Hematopoietic stem cells were isolated by FACS sorting twice as previously described (26). Reanalysis of the cells revealed greater than 95% of the sorted cells were Thy 1. 1^{lo}, Sca^{hi}, Kit^{hi}, Lin^{-}.

Adenoviral vectors. The *bcl-x_S* adenoviral vector, pAdRSV-bcl-x_S, was constructed by cloning a full length *bcl-x_S* cDNA into the pAdRSV vector [25]. This vector contains an RSV promoter and SV40 polyadenylation signal and allows high level expression of inserted sequences. Replication deficient virus was produced in the permissive human kidney 239 cell line containing complementary sub 360 sequences. Vectors were similarly constructed containing cDNA for thymidine kinase (TK)(pAdRSV-TK) or LacZ (pAdRSV-LacZ). Adenovirus infection and β-galactosidase assays were done essentially as previously described [25].

Hematopoietic Progenitor Cell Assays. Infection and viability assays were performed with adenoviral vectors as previously described [25]. For purging experiments, each infection condition utilized 1 x 10^6 hematopoietic cells admixed with 1.5 x 10^4 MCF-7 breast tumor cells that were stably expressing the G418 resistance gene. These cell mixtures were infected for four hours at a range of multiplicity of infection (MOI) from 2,000 to 10,000 viruses/cell in serum-free medium containing 1 ng/mL c-kit ligand and 10 ng/mL IL-3 (R&D, Minneapolis, MN). Following infection, cells were washed of virus and cultured for 48 hours in DMEM media containing 10% fetal calf serum, 10% horse serum, 0.1 U/mL Epo, 2 ng/mL IL-3, 5 ng/mL GM-CSF, and 10 ng/mL c-kit ligand. Cells were harvested and hematopoietic progenitor assays using 1 x 10^4 cells per assay were performed in triplicate as previously described [25]. Adenoviral infection of MCF-7 cells was assayed by determining colony development in the presence of 1 mg/mL geneticin

(BRL/Gibco, Grand Island, NY) to kill normal hematopoietic cells. After two weeks, developing colonies were stained, scored, and photographed as previously described [25].

Murine Bone Marrow Transplants. All mice (C57bl/6, approximately 25 g) were purchased from Charles River (Wilmington, MA), and were used one week after arrival. Marrow cells for transplantation were obtained from the femora of male mice. Donor bone marrow cells from 4 male mice were pooled to yield a total of ~200 x 10^6 unfractionated marrow cells, which was then equally divided into the following groups for treatment with adenoviral vectors containing TK, Bcl-x_S, LacZ, or mock (identical treatment of marrow with adenoviral free reagents), as well as a group treated with TK containing vector followed by gancyclovir. Treated cells were transplanted into recipient female mice (four per condition) irradiated with two doses of γ-irradiation (6 and 5 Gray delivered at 0.134 Gray/min) separated by three hours in order to decrease gastrointestinal tract toxicity. Bone marrow cells were transplanted by injections into either the tail vein or the retro-orbital sinus of anethestised mice. Survival of the recipients was monitored for up to six months to determine long-term reconstitution.

Mouse Y-specific Sequence PCR Detection. Engraftment of transplanted male marrow cells was detected by PCR amplification of male-specific Y chromosome sequences in female recipients. Mouse Y specific PCR primers were synthesized with the sequences: Primer 1- 5' CAGTACCAGTCAGCAATATTTGTTG and Primer 2- 5' TTTCTGTATGCATTGTTTTGTGAGT. DNA was extracted from bone marrow of recipient mice using a previously described method [27], and used as a template in the following PCR conditions: 200 mM Tris-HCl pH 8.8, 250 mM KCl, 35 mM $MgCl_2$, 200 nM each dNTPs, 250 nM each primer, 1mg template DNA, 2.5 U *Taq* Polymerase (Gibco/BRL, Grand Island, NY). Cycling parameters were 94°, 1 min; 55°, 1 min; 72°, 2 min; 25 cycles. The expected amplification product size is 316 bp. Reaction products were analyzed on a 1.5% agarose/TBE gel.

RESULTS

We have previously shown that an adenovirus expressing *bcl-x_S* can be used to selectively kill cancer cells that contaminate bone marrow (25). To better understand this observation, we determined whether murine stem cells express a transgene when infected with an adenovirus vector. To do this, Thy 1. 1^{lo}, Sca^{hi}, Kit^{hi}, Lin^- cells, which are the murine long term repopulating hematopoietic stem cell, were isolated from the bone marrow of c57black/KA mice by four-color FACS [26]. Either 1 x 10^3 hematopoietic stem cells or control neuroblastoma cells were exposed to 2 x 10^3-1 x10^4 β-galactosidase adenoviruses/cell. As previously reported [25], neuroblastoma cells infected with even the lowest titer of virus expressed β-galactosidase. In contrast, hematopoietic stem cells infected with even 1 x10^4 viruses/cell did not express β-galactosidase (Figure 1A). Next, the ability of adenovirus vectors to transduce human hematopoietic stem cells was tested. To do this, CD34+, Thy 1+, Lin- cells were infected with a LacZ adenovirus. Even when exposed to the highest titers of virus, no cells expressed β–galactosidase (Figure 1B). These data suggest that adenovirus vectors based on the RSV Ad5 system do not transduce hematopoietic stem cells and that such viruses could be used to transduce suicide genes into tumor cells contaminating the bone marrow of patients undergoing high dose chemotherapy and autologous bone marrow transplantation.

In order to assess tumor purging effectiveness of the *bcl-x$_S$* vector, *in vitro* cultures of human hematopoietic cells admixed with MCF-7 cells were treated with adenovirus. Our previous data indicated that an MOI of 2000 viral particles per cell was required to kill neuroblastoma cells. In sharp contrast, an MOI of 10,000 was required to completely kill all MCF-7 breast carcinoma cells. Cells from these mixed hematopoietic/MCF-7 cultures were then cultured in methylcellulose assays in order to assess hematopoietic progenitor cell survival. Numbers of CFU-GM colonies were used as a representative measure of progenitor survival. Some non-specific toxicity was noted in these cultures, as at a MOI of 2000, there was a slight decrease in CFU-GM numbers, whereas the CFU-GM colony number decrease was greater at a MOI of 10,000. However, this loss was non-specific as control vectors containing LacZ instead of *bcl-x$_S$* caused similar reductions in CFU-GM numbers (Fig. 2).

In an attempt to overcome the nonspecific toxicity of the *bcl-x$_S$* adenovirus, a combination of *bcl-x$_S$* and thymidine kinase (TK) containing adenoviral vectors was used in order to reduce the effective concentration of the *bcl-x$_S$* vector. Expression of TK in infected cells leads to a sensitivity to the cytotoxic agent gancyclovir, which is added to the culture media after infection. Many cancer cells derived from cells of epithelial cells overexpress a member of the *bcl-2* family. Since *bcl-2* can inhibit - and *bcl-x$_S$* augment - chemotherapy induced apoptosis, a synergistic effect between TK and *bcl-x$_S$* was expected. Following a treatment with gancyclovir, tumor cell killing in TK/*bcl-x$_S$* treated cultures was found to be as effective as the equivalent MOI of *bcl-x$_S$* alone, but with reduced hematopoietic toxicity. Cultures treated with pAdRSV-*bcl-x$_S$* at a MOI of 10,000 had CFU-GM numbers 26% less than cultures treated with a combination of pAdRSV-TK and pAdRSV-Bcl-x$_S$ at a MOI of 5000 of each vector (Figure 2A). A colony replating assay revealed no detectable surviving MCF-7 tumor cells from these cultures (Figure 2B). Importantly, cultures of bone marrow cells mixed with tumor cells were purged equally effectively with either the *bcl-x$_S$* or the *bcl-x$_S$*/TK treatment.

While the above *in vitro* assays indicated the ability of adenoviral vectors to selectively kill tumor cells, we wished to explore the effects of these vectors on the transplantable hematopoietic stem cell. To accomplish this, we used an *in vivo* murine models. The *in vitro* studies showing the inability of adenoviral vectors to directly infect the hematopoietic stem cell were confirmed by *in vivo* transplantation experiments. We determined the effects of adenoviral vectors on murine transplantable stem cells by their ability to reconstitute long-term hematopoiesis following lethal irradiation. Male bone marrow cells were treated *in vitro* at a MOI of 5000 particles per cell, and transplanted into female recipients. Importantly, marrow cells treated with adenoviral vectors containing either TK (with or without subsequent gancyclovir treatment) or *bcl-x$_S$* rescued mice from lethal irradiation, indicating that transplantable stem cells were resistant to adenoviral infection. As expected, control mice receiving mock-infected marrow also survived, while mice receiving no transplant after irradiation had substantially reduced survival. Moreover, both short- (4 weeks) and long-term (six months) hematopoietic reconstitution occurred in these experiments indicating that both the more committed hematopoietic progenitor cells, as well as the hematopoietic stem cell are resistant to adenoviral infection (data not shown). Finally, the contribution of male donor cells to engraftment was confirmed by PCR detection of mouse Y sequences in the female recipients (Figure 3). These data show that male-specific Y sequences are detected in marrow genomic DNA from all of the female survivors six months after transplant.

FIGURE LEGENDS

Figure 1. *Resistance of mouse and human bone marrow stem cells to adenoviral infection.* A). SHEP-1 neuroblastoma cells stain blue, indicating expression of β-galactosidase after infection with pAdRSV-LacZ adenoviral vector at 2 x 10^3 particles per cell. Virtually 100% of the neuroblastoma cells are β-galactosidase positive following infection (top panel). After infection with pAdRSV-LacZ at 1 x 10^4 particles per cell, mouse long-term reconstituting stem cells (black arrows) are uniformly negative for β-galactosidase activity (bottom panel). B) Human hematopoietic stem cells infected with 2 x10^4 lac Z adenoviruses/cell (top panel) and K562 cells infected with 1 x 10^4 LacZ adenoviruses/cell (bottom panel) were stained with x-gal. Note that all of the K562 cells, but none of the normal human stem cells, express β–galactosidase.

Figure 2. A Progenitor cell assays - bar graph B. Photo of colony plate.

Figure 3. *Long term reconstitution of irradiated mouse hematopoiesis with adenovirus infected donor marrow.* Male donor marrow was used to track engraftment in irradiated female recipient mice. After adenoviral infection, marrow was transplanted into lethally irradiated recipients. Genomic DNA was isolated from six month survivors, and subjected to Y specific sequence PCR detection, followed by agarose gel electrophoresis. The expected 316 bp Y derived product is seen in lanes from all long term survivors as well as male DNA positive control. No PCR product is seen in lanes with female mouse, human male, human female, or H_2O negative controls. Mice receiving no transplant after irradiation had markedly lower survival. Labels above each lane indicate the adenoviral construct used to infect the donor marrow prior to transplant.

Task 3. Develop additional markers for molecular detection of occult breast carcinoma.

-3A. Evaluate the specificity and sensitivity of PCR based detection of other mammary specific RNA sequences. No new markers have been detected. The K19 marker has been both sensitive and specific. Any new PCR detection strategies will be based on the identification of novel genes found in tasks 3C and 3D.

-3B. Develop non-radioactive detection schema. A nested primer approach was used to detect K19 cDNA (see appendix, manuscript #1). PCR experiments using K19 primers labeled with either 6FAM or HEX. The PCR products were analyzed with an automatic sequencer. Various amounts of MCF-7 breast cancer cell RNA was mixed with normal bone marrow RNA (from 1x 10^6 cells). These mixing experiments showed that after 2 rounds of PCR we were able to detect K19 mRNA in 2/3 samples containing 10 MCF-7 cells, and 2/4 samples containing 1 MCF-7 cell. K19 was not detected in normal peripheral blood. This task is completed, a non-radioactive detection schema has been developed. Either nested PCR with gel electrophoresis, or with fluorescent detection using an automated sequencer, can be used to detect the K19 mRNA.

-3C. Detection of novel breast cancer peptides. Breast cancer cDNA has been made from RNA isolated from SUM 159 human breast cancer cells. This cDNA will be used to generate a library to identify novel genes.

-3D. As described in the proposal, screening for novel breast cancer peptides will begin this year.

CONCLUSIONS

Significant progress has been made in completing the tasks of this proposal. Keratin 19 appears to be a novel and effective marker for RT-PCR detection of breast cancer cells in peripheral blood and the bone marrow. The collection of patient samples now exceeds one hundred RNA preparations. This includes patients with stage I through IV breast cancer, and includes more than fifty patients that have undergone BMT.

As an adjunct to surgery, radiation, or chemotherapy, autologous bone marrow transplants (BMTs) are increasingly used as a method to increase survival of patients with aggressive non-hematopoietic tumors. However, retroviral tagging and PCR studies indicate that autologous marrow is often the source of cancer relapse in these patients. Several methods have been devised to purge marrow of tumor cells prior to transplantation, but each has distinctive shortcomings. Immunologic methods depend on a unique tumor cell surface epitope and a high avidity antibody for efficient negative selection. Chemical techniques can have significant hematopoietic toxicity. We have previously shown that adenovirally mediated transient expression of *bcl-x$_S$*, a functional repressor of *bcl-2*, would induce PCD in contaminating tumor cells found in bone marrow cell preparations. It is reported here that a pure population of hematopoietic stem cells does not express a transgene when exposed to a recombinant adenovirus. We further postulated that hematopoietic stem cells would retain the ability to repopulate hematopoiesis following treatment with adenoviral vectors.

We show that a combination of *bcl-x$_S$* and TK adenovirus are the most effective and least toxic method of killing MCF-7 cells in a mixed tumor/hematopoietic *ex vivo* culture. Of all tumor cell types we have tested to date, MCF-7 cells have proven to be among the most resistant to *bcl-x$_S$* adenovirus treatment[25]. By combining a TK adenovirus/gancyclovir treatment with *bcl-x$_S$* adenovirus infection of mixed tumor/marrow cultures, MCF-7 cells are purged from the marrow to below detectable levels. In addition, the combination TK/ *bcl-x$_S$* treatment results in an equally efficient purging of hematopoietic progenitors compared to an equivalent treatment using only *bcl-x$_S$*, while resulting in a progenitor cell toxicity that is equal or slightly less. This combined treatment minimizes the non-specific hematopoietic toxicity of these adenoviral vectors, while preserving MCF-7 purging efficiency, although TK treatment alone may be a useful treatment. This data thus supports the hypothesis that *bcl-x$_S$* overexpression mediated by adenoviral vectors may be used to effectively purge solid tumor cells from human bone marrow. Although the mechanism behind this observation is unclear, to date MCF-7 cells have proven to be the only cell type for which a combined *bcl-x$_S$*/TK infection is required to completely purge human marrow *in vivo*. Other cancer cell line, such as the SHEP 1 neuroblastoma line, are efficiently purged by *bcl-x$_S$* viral MOIs that do not have any significant effect on hematopoietic cell activity (25).

In this work, hematopoietic stem and progenitor cells are shown to exhibit resistance to greater MOIs than that required to infect 100% of neuroblastoma cells with an adenovirus marker gene. Previous work has shown that mouse hematopoietic stem cells can be isolated on the basis of the phenotype Thy-1.1^{lo} Lin$^-$ Sca-1$^+$ [30]. As few as 30 of

these cells can rescue 100% of lethally irradiated mice, producing long-term, multilineage reconstitution (26). By purifying homogeneous populations of murine stem cells, we were able to directly target them *in vitro* with adenovirus at MOIs greater than those necessary to kill tumor cells. After such treatment, murine stem cells retained their viability but did not express the adenoviral LacZ gene, whereas tumor cells expressed this marker. Confirming and extending this data, our *in vivo* studies show that murine marrow infected with bcl-x_S, TK, or LacZ adenovirus retains the capacity for long term, apparently multilineage, engraftment upon transplant into lethally irradiated syngeneic mice.

The ability to selectively kill tumor cells, while sparing all of the hematopoietic cells in bone marrow prior to autologous transplantation represents a novel method in purging/transplantation as a treatment of many human neoplasms. Previous methods involving immunologic, mechanical, or chemical based tumor purging have had limited success, require extensive marrow processing, or are useful for treatment of only one specific cell type. We conclude that bone marrow purging using an adenoviral-based method (that can be used alone or in conjunction with other purging strategies) represents a simple, quick, and efficient method for purging a wide variety of non-hematopoietic tumor cells while retaining hematopoietic stem cell activity.

The bcl-x_S adenovirus will begin toxicology testing for the FDA this year. We anticipate clinical breast cancer clinical trials to begin with this virus within one to two years.

Finally, a human breast cancer cDNA has been made and will be used to attempt to identify novel peptides expressed by breast cancer cells. Any such peptides identified will be used to develop new diagnostic markers for breast cancer.

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FIGURE 1

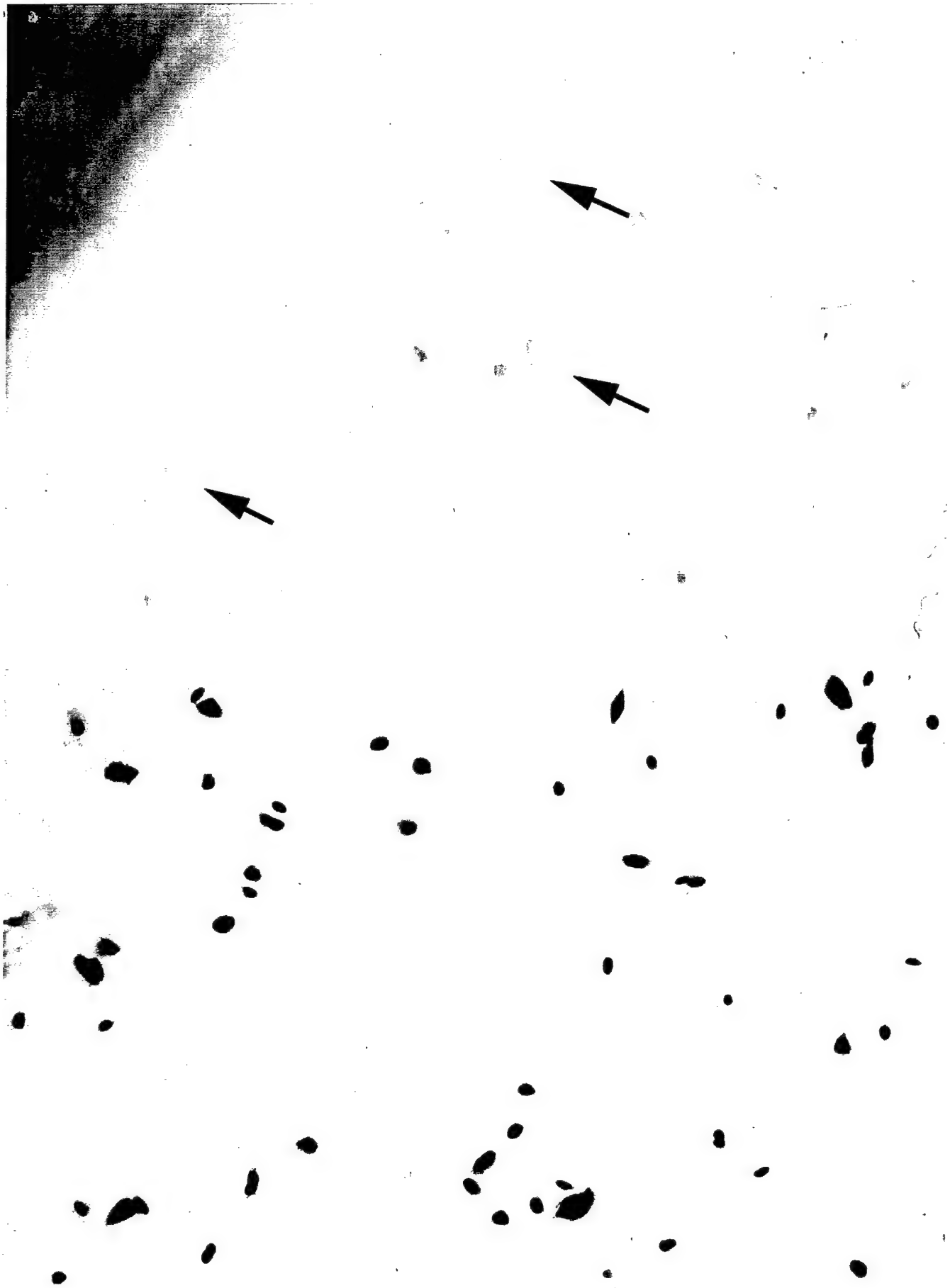


FIGURE 1B

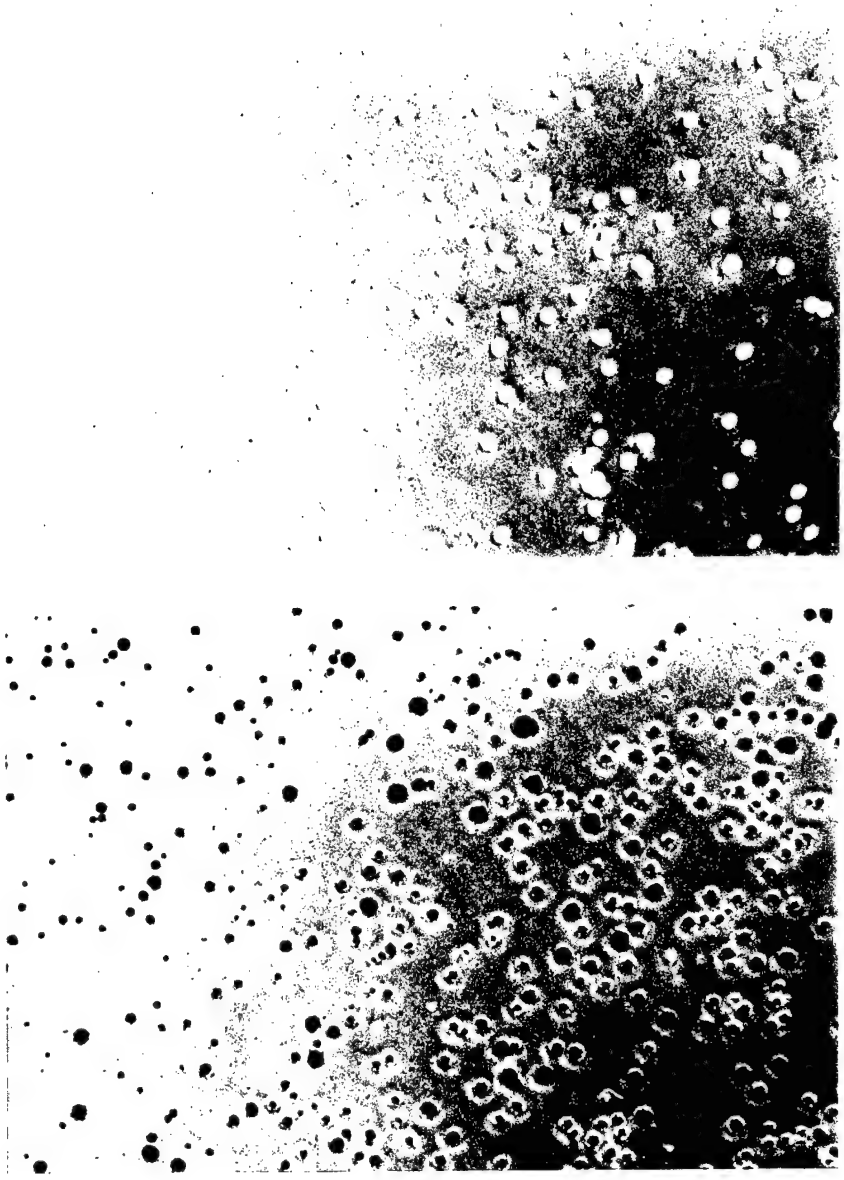


FIGURE 2A

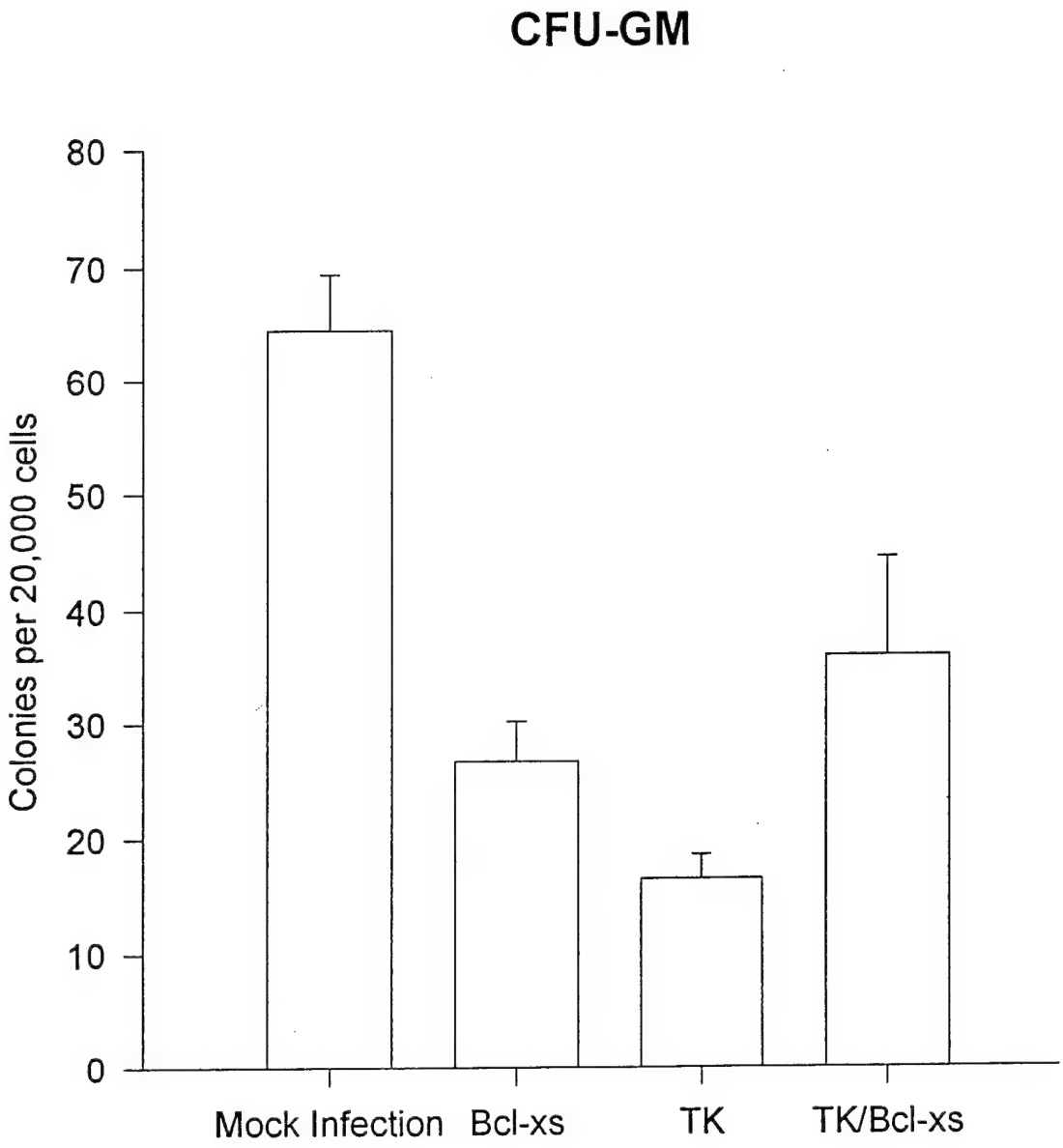

CFU-GM
Colonies per 20,000 cells
80
70
60
50
40
30
20
10
0
Mock Infection
Bcl-xs
TK
TK/Bcl-xs

FIGURE 2B

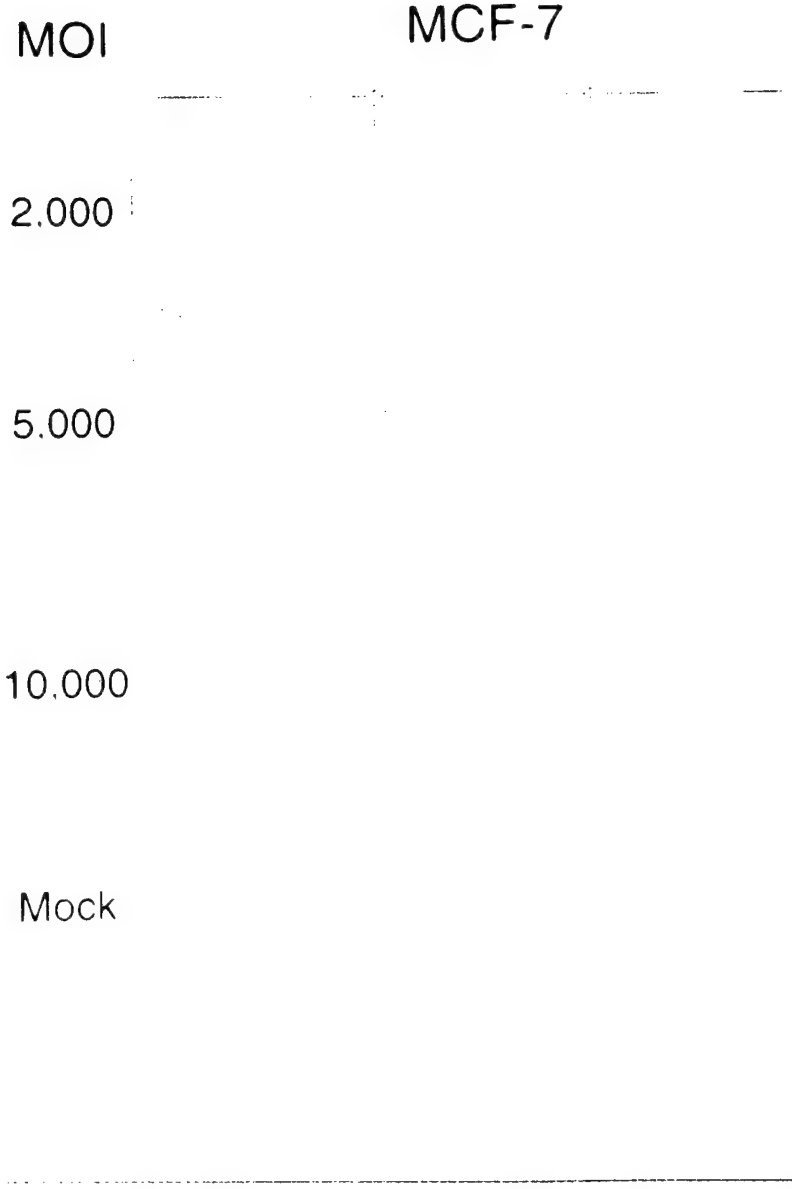
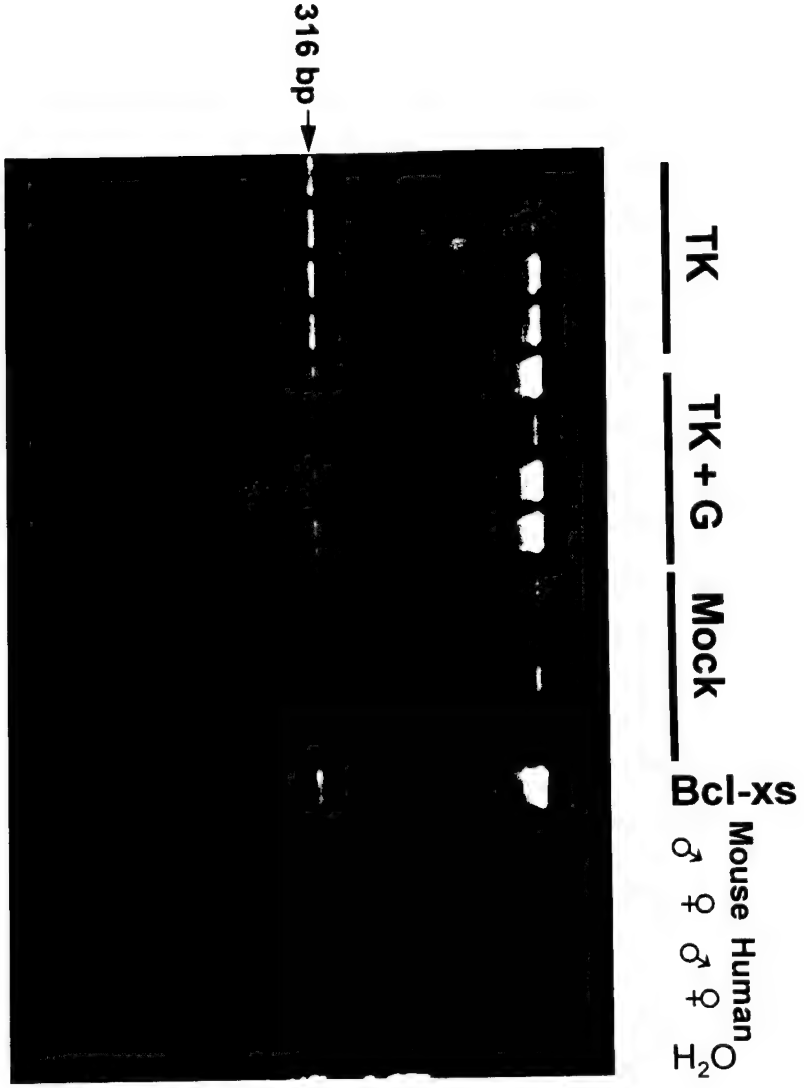


FIGURE 3



The University of Michigan Medical School

Institutional Review Board for Human Subject Research

5560 Medical Sciences Research Building-2, Ann Arbor, Michigan 48109-0678
Telephone: 313 763 4321 • *Telefacsimile:* 313 763 9603 •
Electronic Mail: irbmed@umich.edu • *Internet Web URL:* http://www.med.umich.edu/irbmed •

NOTICE OF APPROVAL OF RESEARCH PROJECT WITH INFORMED CONSENT REQUIREMENT

Clarke, Michael ***(Principal Investigator)*** 1996-562 ***(IRBMED #)***
1996/09/16 ***(Submit Date)*** 1996/11/06 ***(Receipt Date)***
Targeting PCD (programmed cell death) for cancer treatment. ***(Project Title)***
New Project ***(Project Designation)***
Single ***(Application Type)***
None ***(Vulnerable Subject Groups)***
No more than minimal ***(Level of Risk)***
Approved: Consent Required ***(Outcome)***
1996/12/12 ***(Decision Date)*** • 1997/12/12 ***(Expiration Date)***

The Institutional Review Board for Human Subject Research - Medical School (IRBMED) has reviewed the project identified above, and found it to meet all requirements of the ***"Multiple Project Assurance 1184"*** enacted between the University of Michigan and the Office for Protection from Research Risks of the National Institutes of Health, and the ***"Federal Policy for the Protection of Human Subjects"*** of the United States Government. The informed consent process to be used in the project has been found to conform with Federal regulations. The investigators of the project are hereby authorized to proceed with all aspects of the study involving human subjects.

During the course of this study, the investigators are required to meet the following conditions of the approval:

1. Obtain informed consent from each subject, before subject's involvement in the study begins. Document the consent in three copies: one for to the subject, one for investigators' files, and one for the subject's medical record at the University of Michigan Hospitals.
2. On each copy of the informed consent document to be presented to a subject, enter "Approval Date", and "Expiration Date"; they are shown above ("Decision Date" is Approval Date).
3. Report to the IRBMED any planned change in the study, and do not implement any change without receiving approval, except to eliminate immediate hazard to subjects.
4. Report to the IRBMED any unanticipated problems involving risks to subjects.
5. Report to the IRBMED any new information on the project that may adversely influence the risk/benefit ratio.
6. Apply to the IRBMED for continuation of the study well in advance of the "Expiration Date" indicated above, and cease any subject recruitment activity in case the approval is allowed to expire.

To obtain more information on regulations pertaining to human subject research, please browse the Internet Web site of the IRBMED, at the URL location shown in the letterhead.

A list of the members of the IRBMED, who participated in the review process, is appended. It may be submitted to any sponsor of the research.

Sumer Belbez Pek, MD
Professor of Internal Medicine
Chairperson, IRBMED

Copies to:
Principal Investigator
Medical School Assoc. Dean for Research & Graduate Studies
Division of Research & Development Administration

CONSENT FOR EXPERIMENTAL BONE MARROW DONATION
Targeting PCD for Cancer Treatment
NORMAL DONOR

Donor's Name: _____

Registration Number (if applicable): _____

Donor's Address: _____

Social Security Number: _____ Birthdate: _____

I hereby volunteer and consent to participate in a research project being conducted by Dr. Mike Clarke through the facilities of the University of Michigan. Dr. Clarke has talked to me about the research and has given me sufficient time to consider participation. Specifically, the doctor explained to me that:

1. The procedure generally consists of the following: Aspiration of a bone marrow sample(s) and/or blood sample of one or two teaspoons (5-10 mls) per donation.

2. The following discomforts and risks may be involved: Bone marrow aspiration will likely be painful at the moment of aspiration; local anesthesia will be used to reduce discomfort. The site of aspiration may remain tender for several days. There is an extremely low but present possibility of local infection. Participation in multiple studies may be hazardous to you. If you are already participating in another study, please inform us fully. You should not participate in multiple studies, unless you and the investigators agree that your health and the outcome of the study will not be jeopardized. If you are or may become pregnant, this research may involve unforeseeable risks to you, the embryo or the fetus.

3. This research has the following expected benefits to myself or others: As a volunteer donor I will receive $60.00 for the bone marrow donation. My donation will only be used in a laboratory setting. Society, in general, may benefit from knowledge gained in this study, particularly with respect to the care of patients with cancer and of patients undergoing bone marrow transplantation, and with respect to normal bone marrow growth characteristics.

4. I understand that the researcher and the University will not identify me in any write-ups of this procedure and will keep records identifying me confidential to the extent provided by federal, state, and local law.

5. I understand that the University will provide first aid medical treatment in the unlikely event of physical injury resulting from research procedures. Additional medical treatment will be provided in accordance with the University's determination of its responsibility to do so. The University does not, however, provide compensation to a person who is injured while participating as a subject in research.

6. Dr. Clarke has offered to answer any questions I may have concerning the procedure and has explained to me that I may contact him/her at any time (phone 936-5310) for answers to questions about research, my rights, or any injury I may feel is research related. If you have any questions or concerns about your rights as a research subject, you may also contact the Office of Patient-Staff Relations, C246 Med Inn Building, Box 0822, 763-5456.

7. I understand that participation in this project is voluntary and that I may withdraw and discontinue participation at any time without penalty or loss of benefits to which I may otherwise be entitled.

8. I understand that I may be asked to have my blood tested for HIV or Hepatitis in case of a laboratory accident involving my bone marrow.

9. My signature below signifies that I have read this document, understand its meaning, that I have requested a copy of this form if I wish one for my records, and that I freely consent to have this procedure done.

Donor's Signature _____ Date _____

Physician's Signature _____

Witness _____

The University of Michigan Medical School

Institutional Review Board for Human Subject Research

5560 Medical Sciences Research Building-2, Ann Arbor, Michigan 48109-0678
Telephone: 313 763 4321 • *Telefacsimile:* 313 763 9603 •
Electronic Mail: irbmed@umich.edu • *Internet Web URL:* http://www.med.umich.edu/irbmed •

NOTICE OF APPROVAL OF RESEARCH PROJECT WITH INFORMED CONSENT REQUIREMENT

Clarke, Michael ***(Principal Investigator)*** 1992-236 ***(IRBMED #)***
1996/11/07 ***(Submit Date)*** 1996/11/07 ***(Receipt Date)***
Molecular detection of breast cancer. ***(Project Title)***
Previously Approved Project ***(Project Designation)***
Scheduled-Continuation: Single •¤• Amendments: Consent Document ◊ Investigatorship
(Application Type)
No Increase ***(Level of Risk)***
Approved: Consent Required ***(Outcome)***
1996/12/19 ***(Decision Date)*** • 1997/12/19 ***(Expiration Date)***

The Institutional Review Board for Human Subject Research - Medical School (IRBMED) has reviewed the project identified above, and found it to meet all requirements of the ***"Multiple Project Assurance 1184"*** enacted between the University of Michigan and the Office for Protection from Research Risks of the National Institutes of Health, and the ***"Federal Policy for the Protection of Human Subjects"*** of the United States Government. The informed consent process to be used in the project has been found to conform with Federal regulations. The investigators of the project are hereby authorized to proceed with all aspects of the study involving human subjects.

During the course of this study, the investigators are required to meet the following conditions of the approval:

1. Obtain informed consent from each subject, before subject's involvement in the study begins. Document the consent in three copies: one for to the subject, one for investigators' files, and one for the subject's medical record at the University of Michigan Hospitals.
2. On each copy of the informed consent document to be presented to a subject, enter "Approval Date", and "Expiration Date"; they are shown above ("Decision Date" is Approval Date).
3. Report to the IRBMED any planned change in the study, and do not implement any change without receiving approval, except to eliminate immediate hazard to subjects.
4. Report to the IRBMED any unanticipated problems involving risks to subjects.
5. Report to the IRBMED any new information on the project that may adversely influence the risk/benefit ratio.
6. Apply to the IRBMED for continuation of the study well in advance of the "Expiration Date" indicated above, and cease any subject recruitment activity in case the approval is allowed to expire.

To obtain more information on regulations pertaining to human subject research, please browse the Internet Web site of the IRBMED, at the URL location shown in the letterhead.

A list of the members of the IRBMED, who participated in the review process, is appended. It may be submitted to any sponsor of the research.

Sumer Belbez Pek, MD
Professor of Internal Medicine
Chairperson, IRBMED

Copies to:
✓Principal Investigator
Medical School Assoc. Dean for Research & Graduate Studies
Division of Research & Development Administration

CONSENT FOR EXPERIMENTAL BLOOD/BONE MARROW DONATION
Patient with Breast Cancer

Donor's Name: _____

Registration Number (if applicable): _____

Donor's Address: _____

Social Security Number: _____ Birthdate: _____

I hereby volunteer and consent to participate in a research project being conducted by Dr. Clarke to detect breast cancer cells circulating in peripheral blood or in the bone marrow of patients with breast cancer through the facilities of the University of Michigan. A sensitive molecular screening technique will be used to detect breast cancer cells in the blood or bone marrow of patients with breast cancer. The women will be followed routinely, and outcomes will be compared of women with or without circulating breast cancer cells at presentation. My physician has talked to me about the research and given me sufficient time to consider participation. Specifically, the doctor explained to me that:

1. The procedure generally consists of the collection of an extra blood/bone marrow sample of one or two teaspoons (5-10 mls) of blood/bone marrow during a routine blood/bone marrow draw .

2. The donation of this extra blood/bone marrow involves no additional risks.

3. This research has the following expected benefits to myself or others: My donation will only be used in a laboratory setting. Society, in general, may benefit from knowledge gained in this study.

4. I understand that the researcher and the University will not identify me in any write-ups of this procedure and will keep records identifying me confidential to the extent provided by federal, state, and local law.

5. I understand that the University will provide first aid medical treatment in the unlikely event of physical injury resulting from research procedures. Additional medical treatment will be provided in accordance with the University's determination of its responsibility to do so. The University does not, however, provide compensation to a person who is injured while participating as a subject in research.

6. My physician has offered to answer any questions I may have concerning the procedure and has explained to me that I may contact him at any time (phone 764-8195) for answers to questions about research, my rights, or any injury I may feel is research related.

7. I understand that participation in this project is voluntary and that I may withdraw and discontinue participation at any time without penalty or loss of benefits to which I may otherwise be entitled.

8. I understand that I may be asked to have my blood tested for HIV or Hepatitis in case of a laboratory accident involving my blood sample.

9. My signature below signifies that I have read this document, understand its meaning, that I have requested a copy of this form if I wish one for my records, and that I freely consent to have this procedure done.

10. If significant new knowledge is obtained, I will be informed of this knowledge. To find out more about any aspect of this study, including your rights, I may contact the persons whose names, addresses and telephone numbers appear below: Dr. Michael F. Clarke, M.D., University of Michigan Medical Center, Ann Arbor, MI 48109, (313) 764-8195; Bill Spangler, Office of Patient-Staff Relations, (313) 763-5456.

Donor's Signature _____ Date _____

Physician's Signature _____

Witness _____